Journal of Microscopy, Vol. 197, Pt 1, January 2000, pp. 94–100.
Received 25 March 1999; accepted 20 July 1999

Minimal compression of ultrathin sections with use of an oscillating diamond knife

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Key words. Electron microscopy, oscillating diamond knife, ultramicrotome, ultrathin sectioning.

Summary

With the aim to minimize compression artefacts in ultrathin sections, coincident with the stroke direction, we have invented an oscillating diamond knife. Results and theoretical considerations explaining its function are discussed. During conventional ultrathin sectioning the resultant compression is in the order of 20–35% of section height. This holds true for sections of samples embedded into Lowicryl HM20 and of the polymer polystyrene, cut with a 45° diamond knife and floated on water. The oscillating knife reduces this compression almost completely. It consists of a diamond knife on which a low voltage piezoelectric translator (piezo) is mounted, which oscillates when the piezo is driven by an alternating voltage source. No additional cutting artefacts were observed in the micrographs when they were compared with sections produced without oscillating the knife.

Introduction

During ultrathin sectioning of resin-embedded samples, the sections are compressed depending on the sectioning angle (defined as the clearance angle plus the knife angle) and on the properties of the resin. Minimizing section compression is important for every form of morphometry. Cryosections of vitreous samples are even more compressed than the resin embedded samples, i.e. in the order of 30–60% (Richter, 1994). This heavy compression implies that the ultrathin sections are twice as thick as expected from the feed of the sample. In addition to causing structural distortion, section thickening deteriorates resolution. Thus, uncompressed cryosections would represent the native state of the sample much better at the ultrastructural level.

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In the case of diamond knives, the compression of the sections is less severe when the knife angle is reduced (Jésior, 1989). The reduction in compression is about one-third to one-half when a 35° knife angle is used instead of a 45° knife angle, but still the sections are compressed. Because sample compression correlates with the knife angle or the sectioning angle, one may suggest that the knife angle should be reduced as much as possible. However, the reduction in knife angle is limited by the susceptibility of the knife to damage at small angles. In practice, knife angles smaller than 30° are not used; thus, this straightforward adjustment to reduce compression is limited. The next possibility is to reduce the sectioning angle (without reducing the knife angle!) by tilting or oscillating the knife edge. A theoretical background was given many years ago (Olbrich, 1947). The improved quality of tissue sections (about 20 μm thick) by the use of vibratomes was documented long ago (Smith, 1970; Sitte & Neumann, 1983). We propose that the reduction of the sectioning angle in the case of the oscillating knife depends on the cutting speed of the sample and the amplitude and the frequency of the knife edge. We explain theoretically how the sectioning angle is influenced by tilting or oscillating the knife. Furthermore, we show that ultrathin sections produced with an oscillating diamond knife are almost uncompressed without exhibiting additional cutting artefacts, and we discuss some useful applications of the oscillating diamond knife.

Methods

Sample preparation

A transparent polystyrene sample was trimmed and ultrathin sectioned. The sections were observed in the light microscope. Dinoflagellates, the protozoan *Amphidinium carterae*, were fixed with 2% paraformaldehyde/0·2%

glutaraldehyde, dehydrated with ethanol and embedded in Lowicryl HM20 (Chemische Werke Lowi, Waldkraiburg, Germany) at −30 °C according to the progressive lowering of temperature (PLT) technique (Carlemalm *et al.*, 1985). These samples were poststained with uranyl acetate and lead citrate (Reynolds, 1963) after sectioning. The samples were investigated by light and electron microscopy.

Oscillating diamond knife

The oscillating diamond knife (Fig. 2a,b) consists of a diamond knife with a knife angle of 45° (Diatome, Biel, Switzerland) equipped with an oscillator (Fig. 2a) at the distal end of the piezo a counterweight was fixed for some experiments. As oscillator we used in our set-up a low voltage piezoelectric translator (piezo; PI, Waldbrunn, Germany). A power supply of variable alternating voltage and frequency was connected to the piezo. The values were checked with an oscilloscope. The oscillating mass of the piezo actuated the knife.

Sectioning conditions

Polystyrene was sectioned with a relatively high amplitude (400 nm) and a low frequency (2000 Hz) of the oscillator. For improving transmission of oscillation under these conditions, a counterweight of 10 g was fixed at the distal end of the piezo (Fig. 2a). For sectioning *A. carterae* embedded in Lowicryl HM20, the unmodified piezo was connected to the knife and driven with an amplitude of 150 nm and a frequency of 16 kHz during sectioning (under these conditions, no counterweight had to be attached to the piezo's distal end to achieve decompression of the sections). The sections were cut with an Ultracut S ultramicrotome (Leica, Vienna, Austria). Cutting speed was varied in the range 0·05–0·2 mm s^{-1}. The value for optimal alternating voltage (± 1 V) and frequency (16 kHz) applied to the piezo was found empirically by varying the settings and evaluating resultant sections.

Theoretical considerations

Some of the angles playing an important role during sectioning are defined in Fig. 1. The sectioning angle (α) corresponds to the knife angle (κ) plus the clearance angle (τ) when the stroke hits perpendicularly to the knife edge (the tilt angle γ_1 is zero). The most straightforward way of reducing α is to reduce the knife angle (κ). Alternatively, tilting the stroke by γ_1 in the plane (PP_2P_3) of the block face will also reduce the apparent sectioning angle α_2 (note that the result is the same whether the stroke or the knife is tilted by γ_1 in plane PP_2P_3). The following trigonometric expressions define the geometry of the tilt angle γ_1, and

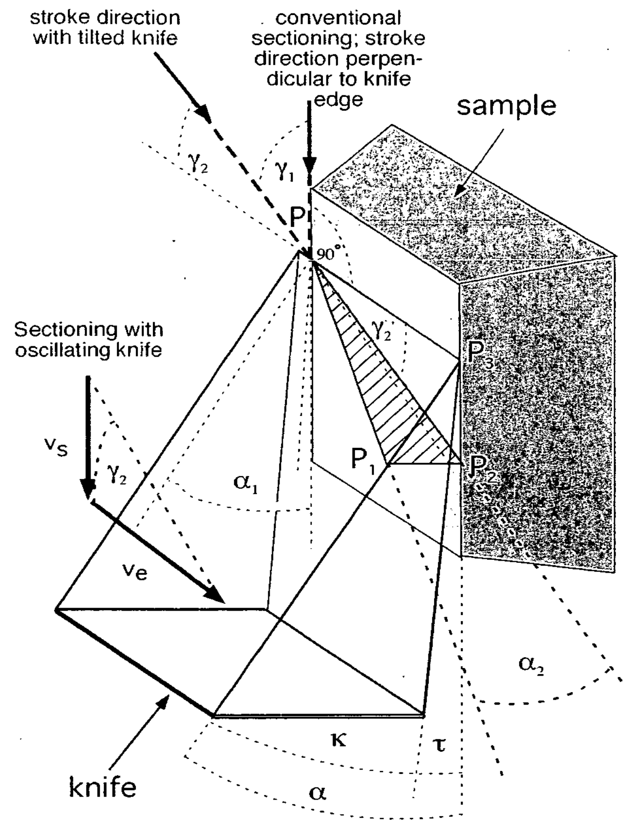


Fig. 1. Schematic drawing showing the correlation between knife, clearance, tilt and sectioning angle during ultrathin sectioning. Knife edge and sample blockface are always located in the plane defined by points PP_2P_3. When the stroke with the sample hits the knife edge perpendicularly (tilt angle $\gamma_1 = 0°$), the sectioning angle (α) corresponds to the knife angle (κ) plus the clearance angle (τ). When the stroke hits the knife edge at an angle γ_2 smaller than 90° (γ_2 and $\gamma_{2'}$ correspond to $(90° - \gamma_1)$), the resulting sectioning angle (α_2) becomes smaller than α. α also becomes smaller when the knife edge is oscillating (mean velocity of the edge v_e) in plane PP_2P_3 perpendicular to the stroke (stroke velocity v_s). The smaller the ratio v_s/v_e is, the smaller becomes the sectioning angle (α_2). The mathematical relations between γ_2, penetration/oscillation (v_s/v_e), α, and α_2 are given in the theoretical considerations.

the sectioning angles α and α_2:

$$\gamma_1 + \gamma_2 = 90° \tag{1}$$

therefore

$$\gamma_2 = (90° - \gamma_1) \tag{2}$$

and

$$\gamma_2 = \gamma_{2'} \tag{3}$$

$$\tan \alpha_2 = P_1P_2/PP_2 \tag{4}$$

$$\sin \gamma_{2'} = P_2P_3/PP_2 \tag{5}$$

$$\tan \alpha = P_1P_2/P_2P_3 \tag{6}$$

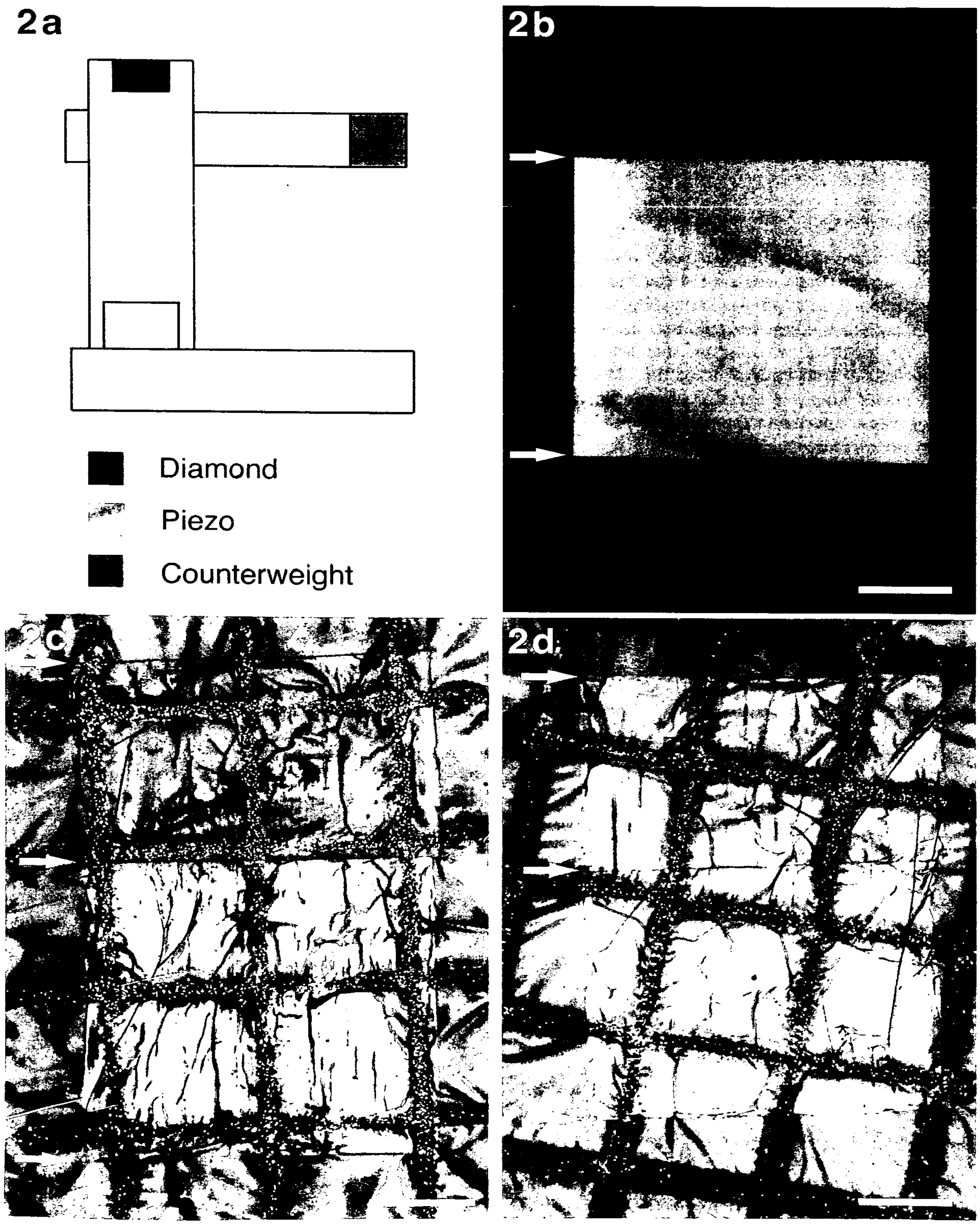

2a
Diamond
Piezo
Counterweight
2b
2c
2d

Table 1. Compression of ultrathin sections under various conditions

Sample	Knife-angle (°)/sample speed ($mm\,s^{-1}$)	Vibration: amplitude (nm)/frequency (Hz)	Mean cutting angle (°)	Compression: section/blockface (%)
HM20	45/0·1	0/0	45	22·5
HM20	45/0·1	150/16000	2·5	7·5
Polystyrene	45/0·05	0/0	45	35·0
Polystyrene	45/0·05	400/2000	4·4	3·5
Polystyrene	45/0·2	400/2000	18	18·0

Multiplying (5) and (6) and substituting the product into (4) we arrive at (7):

$$\tan\alpha_2 = \sin\gamma_{2'}.\tan\alpha(\mathrm{P_1P_2/PP_2 = P_2P_3/PP_2.P_1P_2/P_2P_3}) \quad (7)$$

The sectioning angle α (at zero tilt), hence $\tan\alpha$, is constant because the knife angle (κ) and clearance angle (τ) are defined for any given knife in ultramicrotomy. Thus the sectioning angle α_2, resulting from tilting the stroke, depends mainly upon $\gamma_{2'}$. It follows from Eq. (7) that decreasing $\gamma_{2'}$ (following Eqs (1) to (3) therefore γ_1 is increasing) results in the reduction of the apparent sectioning angle α_2. When the tilt angle γ_1 approaches 90° the apparent sectioning angle becomes vanishingly small. Using the small angle approximation, the trigonometric relations mean that $\sin\gamma_{2'}$ becomes $\tan\gamma_{2'}$ and $\tan\alpha_2$ becomes α_2. Therefore, for very small $\gamma_{2'}$ the apparent sectioning angle can be defined as:

$$\alpha_2 = \tan\gamma_{2'}.\tan\alpha$$

$$\text{(holds for small } \gamma_{2'} \text{ and large tilt angles } \gamma_1) \quad (8)$$

In practice, as will be discussed later, tilting the stroke, which is equivalent to tilting the knife blade, causes severe problems in ultramicrotomy. As an alternative to tilting the knife one can oscillate it. By way of analogy, this is what we experience when cutting bread by moving a knife back and forth. Imagine that a long knife is being used to slice a loaf of bread and to cut the whole loaf there is only one translational movement necessary. In this case γ_2, hence $\gamma_{2'}$, are defined by the penetrating speed, v_s, and the moving speed, v_e. v_s is the speed with which the knife cuts into the bread and v_e is the translation velocity with which the knife is pushed or pulled. The vectorial relationship between these velocities defines $\gamma_{2'}$, as demonstrated in Fig. 1:

$$v_s/v_e = \tan\gamma_{2'} \quad (9)$$

The apparent sectioning angle is redefined in terms of cutting and knife edge speed by inserting (9) into (8):

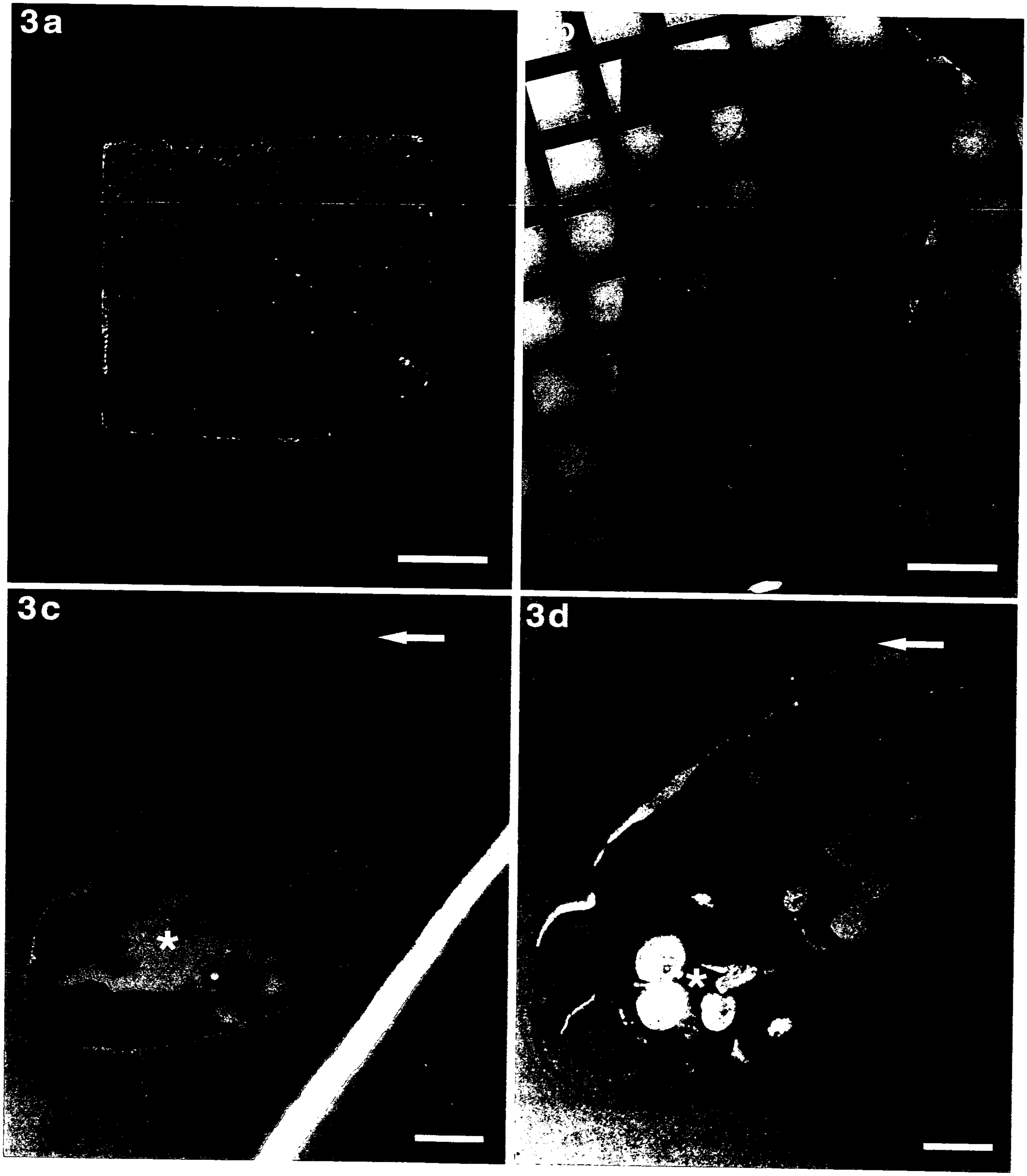
$$\alpha_2 = v_s/v_e\cdot\tan\alpha(v_e \gg v_s; \tan\alpha = \text{constant}) \quad (10)$$

Equation (10) is only correct for small values of the quotient v_s/v_e. This means that v_s has to be very small compared to v_e. When the knife is reciprocating, or oscillating, the knife edge is changing direction at a certain frequency and the speed of the knife is not constant, alternating between 0 and $v_e$max. The mean speed (v_e) of the knife edge, however, is defined as $A\cdot\nu$, where A is the amplitude and ν is the frequency of the oscillation of the knife. When we consider a knife angle κ of 45°, a clearance angle τ of 6°, an oscillating amplitude of 400 nm, a frequency of 2 kHz (resulting in a mean speed v_e of $0{\cdot}8\,mm\,s^{-1}$) and a cutting speed of $0{\cdot}05\,mm\,s^{-1}$, the mean sectioning angle (α_2) is about 4·4°. Of course, this is a mean value for the apparent sectioning angle α_2 which oscillates between 51° and about 2·2°, because the speed of the knife edge is changing from 0 to $v_e$max ($= 2v_e$).

Results

The following two examples demonstrate how ultrathin sectioning can be improved by the use of an oscillating diamond knife.

Fig. 2. (a) Schematic depiction of the oscillating diamond knife. The knife is shown as a projection looking from the sample blockface to the knife. Two thin supports connect the knife to a stable base. With this design the oscillation of the piezo vibrates the knife. The counterweight was only used to cut polystyrene. At low frequencies and high amplitudes of the piezo this helps to make the knife more efficient. (b) The blockface of a cut polystyrene sample. (c) The difference in compression when the sample is sectioned without (upper section) or with (lower section) oscillation of the knife. The lower section suffered almost no compression whereas the upper one shows 35% compression. (d) Essentially the same as (c), but the coefficient v_s/v_e was raised by increasing the stroke velocity of the sample by a factor of 4; this resulted in a compression of 18% (d) compared with only 3·5% compression in (c). (b)–(d) Images taken in reflected light mode. Arrowheads point to front and rear end of the block (b) and of the sections (c,d), respectively. Bars represent 100 μm.


3a
3c
3d

Sectioning of polystyrene

Polystyrene was sectioned with a piezo carrying a 10 g counterweight. The counterweight made it possible to actuate the piezo with a relatively low frequency (2 kHz), but with a relatively high amplitude (2·6 V: corresponding to a piezo amplitude of 400 nm). With the use of polystyrene we could demonstrate that the sectioning angle has a large influence on the occurrence of compression artefacts. When we changed the stroke velocity by a factor of 4, corresponding to a change of the mean sectioning angle from 4·4° (Fig. 2c) to about 18° (Fig. 2d), the increase in compression was fivefold (18% in the lower section shown in Fig. 2d compared to 3·5% of the lower section shown in Fig. 2c; see also Table 1). No oscillation of the knife (sectioning angle 51°) resulted in a compression of 35% (upper sections in both Fig. 2c,d). All these sections were compared with the size of the blockface shown in Fig. 2b.

Sectioning of HM20-embedded dinoflagellates

For samples embedded in Lowicryl HM20, sectioning with the 45° diamond knife, without oscillation, resulted in a compression of about 22·5% (Table 1; Fig. 3b upper section, Fig. 3c). The compression was significantly reduced when the knife was oscillated (7·5%; Fig. 3b lower section). Optimal results were achieved when the piezo was actuated with a voltage of ± 0·5 V (amplitude 1 V), corresponding to a piezo amplitude of 150 nm and a frequency of 16 kHz. The structures were barely compressed when the knife was oscillating and astonishingly there were no vibration artefacts (scratches) on the sections (Fig. 3d). However, knife marks were visibly wider when the knife edge was oscillating (arrows in Fig. 3c and d). The cell shown in Fig. 3c and d was chosen because the embedding was not optimal and therefore the sample was very difficult to cut. The difference in sectioning quality is clearly demonstrated. Conventional non-oscillating sectioning resulted in destruction of fine structures (located close to the asterisk, Fig. 3c); these intracellular vesicles were obviously not well embedded in HM20. It seemed that the embedding polymer was not optimally crosslinked and therefore the stability of the section was weak. When the knife oscillated, the weak patches in the sample are well preserved (asterisk, Fig. 3d); the stress induced by compression during sectioning was obviously reduced.

We would like to emphasize that with samples embedded in polymers, compression of sections strongly depends on the embedding protocol ('hard' or less compressible vs. 'soft' embedding), on the age of the embedding material (the older the resin the less compressible: range of years) and the composite mechanical properties. Lowicryl K4M, for example, is a very hydrophilic resin which usually expands on the water surface after sectioning, resulting in dilated sections. Also with K4M we found that the sections were longer in the stroke direction when cut with an oscillating knife than with an immobile knife (result not shown).

Discussion

Reduction of the knife angle and hence the sectioning angle has been demonstrated to reduce significantly the compression of ultrathin sections during the sectioning process (Jésior, 1989). As an alternative method, we prepared sections with extremely tilted knives (tilting angle 75°, and $\gamma_{2'} = 15°$). However, the quality of sections from greatly tilted knives are poor. The sections curl severely during the sectioning process, and one has to continually refocus the stereo microscope during the sectioning stroke because the focal length of the microscope is too short at convenient magnifications. This makes the work impractical. We were unsuccessful in producing quality sections.

The successful alternative was to oscillate the knife. In our calculations we assumed that the oscillation of the knife was identical to the oscillation of the piezo. At present, we do not know the exact amplitude and frequency of the knife during oscillation. However, the fact that the knife does oscillate is evident from three observations. Firstly, without actuating the piezo there are large compressions of the sections compared to those where the piezo is actuated. Secondly, change in stroke velocity (and hence in the mean apparent sectioning angle) induces a significant change in compression. Thirdly, knife marks were widened when the knife edge was oscillated.

At present, we aim to improve the design of the oscillating diamond knife in order to have a more defined oscillation and to know its amplitude. We are also working on a cryo version of the oscillating diamond knife, because cryosections of vitreous material also suffer from compression artefacts (Richter, 1994). We would like to develop these methods because uncompressed ultrathin cryosections of vitreous

Fig. 3. Ultrathin sections of dinoflagellates embedded in HM20. (a) and (b) show the blockface of the HM20 sample after sectioning and the sections on the grid, respectively. The upper section was sectioned conventionally (compression 22·5%), the lower one with the oscillating knife (compression 7·5%). a and b are images taken in reflected light mode. Bars represent 100 μm. c and d show electron micrographs of the same cell. That in (c) is heavily compressed and distorted. The poor embedding of this sample does not withstand conventional sectioning; the intracellular vesicles (*) are distorted. In (d) the cell is barely compressed and adequately preserved (*). By sectioning with the oscillating diamond knife the stress during sectioning is reduced to an amount that the weak areas in the section are maintained. Arrows point to a knife mark. The direction of the knife mark indicates the sectioning direction. Note that the mark is wider in (d) than in (c) because of the oscillation of the knife. Bars represent 1 μm.

samples would reveal structural details of tissues much more closely to the living state than any other approach.

Acknowledgements

We would like to thank Laurée Salamin Michel for the HM20-embedded protozoan, Matthias Chiquet and Gregory Allen, M. E. Müller Institute of Biomechanics, University of Bern, for helping with the manuscript and Jacques Dubochet for supporting Daniel Studer.

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